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Skin test reagents in the diagnosis of atopic disease.

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Summary

The overall aim of this study was to optimize the diagnostic value and safety of the intracutaneous (ICT) and skin prick test (SPT) and to apply the optimized skin test for the determination of the age-dependency and prevalence of skin sensitization to different allergens in a Dutch patient population.

Chapter 2 deals with the stability of histamine solutions. The aim of this study was to investigate the stability of ready-made solutions containing human serum albumin (HSA) or glycerol and phenol, produced under sterile conditions. We developed an anion exchange HPLC method that does not depend on a derivatization technique and in which phenol and histamine could be separated. In this study we used histamine phosphate in concentrations of between 32 and 0.005 mg/ml dissolved in phosphate buffered saline (PBS) with HSA and phenol and histamine dihydrochloride in concentrations of 10, 3 and 1 mg/ml dissolved in PBS with glycerol and phenol, produced under sterile conditions. The histamine and phenol content was determined in all solutions during 1 year of storage at 6° and 25°C using a stability-indicating anion exchange HPLC method separating both compounds, in order to test the stability of these solutions. In addition, the sterility and pH was determined. It can be concluded that all histamine concentrations higher than 0.005 mg/ml are stable for at least 1 year, both at 6° and 25°C. Phenol is surprisingly unstable in the PBS formulation with glycerol and should be omitted.

In chapter 3 the stability of freeze-dried allergen extracts, which are intermediate products (IMP) for the production of final products, such as used for ICT and SPT, was determined. This IMP is often stored for a longer period at -20°C. The aim of this study was to test the accelerated stability method using freeze-dried extracts of timothy pollen, birch pollen, *D. pteronyssinus* and cat dander, which were stored for up to 2 years at elevated temperatures. The relative potency was determined with the enzymallergosorbent test (EAST) inhibition method and the protein composition was evaluated with sodiumdodecylsulfate polyacrylamide gelelectrophoresis (SDS-PAGE). The Arrhenius equation was calculated using the conventional method of linear regression analysis and the method of non-linear regression. This equation was used to predict the stability of such preparations at -20°C.

The freeze-dried extracts of timothy pollen, birch pollen and *D. pteronyssinus* showed a biphasic decrease of relative potency at elevated temperatures, whereas

cat dander showed no decrease of relative potency at any temperature. In general the calculation method using the non-linear model predicted relative potencies closest to the real time values and predicted a lower and more accurate % loss per year at -20°C as compared with the linear regression model. It was concluded that the accelerated stability studies using the method of likelihood ratios for calculation of the Arrhenius equation are probably valid for the prediction of the stability of freeze-dried allergen extracts.

In chapter 4 the influence of temperature, dilution and preservatives on the stability of bronchoprovocation, intracutaneous and skin prick test solutions of allergen extracts of timothy pollen, birch pollen, *D. pteronyssinus* and cat dander was determined using EAST inhibition and SDS-PAGE. In this stability study the ICT method was compared with the EAST inhibition method using different concentrations of standardized timothy pollen, birch pollen, *D. pteronyssinus* and cat dander extracts during 24 months of storage at 6°C. The influence of temperature on various formulations of the above mentioned extracts was determined using the EAST inhibition technique during storage for a maximum of 36 months.

Most formulations were found to be stable for 24 (ICT method) or 36 (EAST inhibition method) months at 6°C. At 25°C most formulations showed a decrease in relative potency, which remained above the acceptance limit of 0.3 times the in-house-reference for the bronchoprovocation formulation of timothy pollen, birch pollen and *D. pteronyssinus* and for the skin prick test formulation of cat dander. It was concluded that cat dander was remarkably stable at 6° and 25°C in glycerol and birch pollen was very susceptible to degradation with phenol. This destructive effect of phenol could be prevented by adding HSA.

The discrepancy between in vivo and in vitro tests reported by others was confirmed for *D. pteronyssinus* and timothy pollen.

It was not possible to determine the stability of the intracutaneous test concentration of timothy pollen and *D. pteronyssinus* using EAST inhibition, because the potency of these dilute extracts was below the detection limit of this method.

In chapter 5 the use of a concentration procedure prior to a normal EAST inhibition is described and validated. Using this procedure the stability of very dilute allergen extracts as used for ICT and immunotherapy could be determined. This procedure was validated and used in a study of the stability of extracts of timothy pollen, birch pollen, *D. pteronyssinus* and cat dander. The stability was determined after storage for 6 and 12 months at 6° and 25°C of a concentration of 30 BU/ml. There was no difference in relative potency before and after concentration of birch and timothy pollen. *D. pteronyssinus* and cat dander showed a significant decrease

of 25% and 35% of the relative potency after concentration. The mean coefficient of variation of 12 determinations of the stability study was 11.8%. For all allergens the 30 BU/ml or appr. 0.00025 mg/ml solution was stable for 12 months at both temperatures, except for *D. pteronyssinus* which declined rapidly at 25°C.

In chapter 6 the optimum concentration and cutoff value of ICT and SPT was determined in a small population using a multiple regression technique for an optimal predictive value of the skin test when compared with the RAST. In this study *Phleum pratensis* and *D. pteronyssinus* were used as test allergens in a multivariate design in order to determine the optimum sensitivity and specificity of the SPT and ICT in relation to the concentration of the allergen preparations, as well as the cutoff limit of the skin tests.

If a RAST value of class 1 or more was taken as an indication of sensitization, it was found that the optimum concentrations for the detection of sensitization are in the range of 10-100 BU/ml and 1,500-10,000 BU/ml for ICT and SPT, respectively. The skin test results were expressed as histamine ratios. Using allergen concentrations of 30 and 3,000 BU/ml, cutoff values of 0.87 and 0.53 and predictive values of 87.1% and 79.1% for ICT and SPT, respectively, were found. The maximum wheal size (=mean wheal size + 2 S.D.) to be expected in 95% of the population was 26.6 mm (ICT) and 10.9 mm (SPT), which is regarded as safe by most clinicians.

In conclusion, using this methodology with a limited number of patients, it is possible to improve the diagnostic precision and safety of the skin test. In the second part of this study these hypotheses were prospectively tested in a multicenter study.

In chapters 7 to 10 the results of a multicenter trial are presented. In this trial, patients, newly referred to specialists of six (ICT) and six other (SPT) allergy centers in The Netherlands, were selected for the ICT (497) or SPT (361). A paediatric clinic was also included in which children were selected for the ICT (103). An allergen-specific clinical history was recorded prior to skin testing and graded as: 0) negative; 1) doubtful; 2) positive. When possible, specific IgE determinations were performed for *D. pteronyssinus*, grasses, tree pollen and cat dander by Phadebas RAST (Pharmacia AB, Uppsala, Sweden).

In chapter 7 the optimum concentrations of allergen and the cutoff values of ICT and SPT, calculated in the first part of the study (chapter 6), using two identical (grass pollen and *D. pteronyssinus*) and two other standardized allergens (tree pollen and cat dander) in the same concentrations were tested. The hypothesis we

set out to test in this second part of the study was that the diagnostic precision for the detection of allergic sensitization and the safety of the skin tests could be predicted in a large, multicenter patient population, based on the results of a small patient population and a limited number of allergens, studied in part I.

The optimum cutoff value of 0.7 (ICT) and 0.4 (SPT) resulted in a predictive value for the detection of allergic sensitization of 83% (RAST) and 77% (CH), and 91% (RAST) and 86% (CH), for the ICT and SPT, respectively. No systemic side effects of the skin tests were recorded.

These overall results correspond well with the predictions regarding safety and predictive value of part I of this study, in which a limited number of patients was studied.

In conclusion, using a limited number of standardized allergens in a small group of patients, it is possible to predict a safe and efficacious concentration for routine skin testing and to extrapolate these results to other standardized allergens.

In chapter 8 the results of the histamine control solution are presented. The reproducibility of both skin tests was calculated in this multicenter study and we examined whether the results of the skin test used in a multicenter setting should be expressed as a mean wheal size or a wheal index by dividing the allergen wheal size by the histamine wheal size.

We demonstrated that the ICT results of the histamine solution are more comparable between the different centers and that the coefficients of variation of the ICT (15.9%) are a factor 2 lower as compared with the results of the SPT (27.2%). Although the SPT has some practical advantages and provokes less side effects, the ICT should be preferred above SPT, when performing a multicenter study, because of a higher reproducibility within and between centers and the results should be expressed as a wheal index.

In chapter 9 the prevalence of sensitivity to allergens as determined using the ICT or SPT and the correlation between sensitization for different allergens was determined. From this, allergens were selected which would be useful in a screening panel for the detection of sensitization to aero-allergens.

Of the standardized allergens, the prevalence rates of sensitization found were 43% and 33% (*D. pteronyssinus*), 28% and 24% (grasses), 15% and 11% (trees), 24% and 22% (cat Dander), 21% and 23% (dog dander) and 7.2% and 9.5% (horse dander), for ICT and SPT respectively. The strongest correlations were found between trees and grasses; epithelia and *D. pteronyssinus*; *D. pteronyssinus* and storage mites and between the moulds. In this out-patient population grasses and *D. pteronyssinus* were the most prevalent allergens, also showing the highest

number of mono-sensitized patients. It is suggested that a small screening panel for atopy should, then, consist of grasses, *D. pteronyssinus* and dog dander.

In chapter 10 the age-dependency of the skin test (ICT) sensitization from childhood to old age (4-75 years) was determined using 6 standardized and 1 non-standardized allergen (*Cladosporium herbarum*). Furthermore, the age-dependency of the skin reactivity to histamine was determined.

It was found that the histamine skin reactivity rose significantly ($p < 0.05$) during childhood, was significantly higher in the age group of 10-15 years, and was constant from 15 to 75 years of age.

The mean wheal index (histamine ratio) of all allergens was constant during childhood, and decreased for grass pollen and *D. pteronyssinus* after the age of 25, and after the age of 15 for the other allergens.

The prevalence of a positive skin test decreased with age, except for grass pollen. During childhood the indoor allergens, cat dander and *D. pteronyssinus*, were the most important allergens, while after the age of 15, the sensitivity to an outdoor allergen, grass pollen, increased markedly. At every age, *D. pteronyssinus* was the most important allergen. After the age of 25 the prevalence of sensitization to all allergens declined. The prevalence of a positive skin test to *Cladosporium* was unexpectedly high in childhood (10-40%).

It was concluded that the prevalence of a positive skin test declined with age, except for grass pollen. The degree of sensitization in asthmatics peaked between the ages of 20 and 40 and the sensitivity to indoor allergens developed earlier than the sensitivity to outdoor allergens.

Stable histamine solutions, standardized allergen extracts with a documented stability, an optimized concentration and skin tests with a documented cutoff value and reproducibility, thus ensuring a good predictive value and safety are a prerequisite for epidemiological studies. These studies show that *D. pteronyssinus* and grass pollen are the most prevalent sensitizing allergens showing the most mono-sensitized patients.